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# Skin Depigmentation Profiles of Ethanolic Fraction of the South African "Green" *Cyclopia intermedia* and Other Related Biological Potentials

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## Abstract

Background: The process of aging in human is complex with underlying multiple influences including the probable involvement of free radicals and varying degree of environmental factors. Over accumulation of such free radicals and other related factors resulted into cellular oxidative damage to important macromolecules such as proteins, lipids and deoxyribonucleic acid eventually leading to many chronic diseases like cancer, diabetes, premature skin aging, atherosclerosis and neurodegenerative disorders. *Cyclopia intermedia* (honeybush) is notable in South Africa and globally for medicinal (including alleviate heartburn and nausea, stimulate milk-production in breast-feeding women and treat colic in babies) and economic importance such as in worldwide production of tea and beverages. This study is therefore directed towards searching for biological values of honeybush in the area of possible combating with cellular oxidative stress and alleviating early skin aging. Method: An ethanolic extract of the South African fynbos species *Cyclopia intermedia* was successively fractionated in an open column of silica using a gradient of hexane: ethyl acetate. Phytochemical constituents of the most bioactive column fraction (VIII) was profiled using chromatographic and LC-ESI-MS methods with notable phenolics as well as unidentified analogues thereof. Result: The biological profile of fraction VIII assessed using colorimetric assays gave potent anti-tyrosinase activity $IC_{50}$ 28.125 µg/mL, while the *in vitro* total antioxidant capacities showed VIII with ORAC (3218.72 ± 90.14 µmol TE/g); TEAC (9903.21 ± 161.73 µmol TE/g); FRAP (6146.38 ± 5.72 µmol AAE/g) and iron (II)-induced microsomal lipid peroxidation estimated as $IC_{50}$ 260.173 µg/mL. Conclusion: The outcome of this investigation suggested more unexplored phenolics are still in existence in *C. intermedia* with possible correlation between the anti-tyrosinase of fraction VIII to the total antioxidant capacities demonstrated.

## Keywords

*Cyclopia intermedia*, Chromatography, Tyrosinase, Phenolics, Antioxidant

## 1. Introduction

Over expression of tyrosinase in a dermatological environment may lead to various degrees of pigmentation-related disorders, usually associated with unpleasant changes to the structural and physiological integrity of the skin [1, 2]. Excess production of melanin has been linked to various dermatological disorders such as melasma, senile lentigo, post-inflammatory melanoderma [3], freckles, age spots and sites of actinic damage which can give rise to esthetic problems [4]. Natural phytochemicals have been proposed as a source for novel, safer and more effective depigmenting agents [5]. In another sector, tyrosinase has also been shown to regulate enzymatic browning of perishable agricultural products thereby causing depreciation in nutritional quality and economic loss of such products [6, 7]. Although, arrays of noteworthy tyrosinase inhibitors have been discovered over the years, regrettably, the effectiveness of some of these products is lacking due to the accumulation of adverse effects including carcinogenic [8, 9], skin irritation, mutagenic, cytotoxic [10], poor skin penetrations and low stability [11].

*Cyclopia intermedia* (Family: Fabaceae, Tribe: Podalyrieae) is an economic plant of South Africa, notable for the production of a commercial herbal tea, honeybush and various extracts [12]. Many of the biological activities

displayed by *C. intermedia* have been proposed to be ascribed to its unique combination of polyphenolic constituents [13]. The bio-activities include, inhibition of skin tumour promotion, $FB_1$-induced liver carcinogenesis [14], oesophageal papillomas [15], photo-protection against UVB-induced skin damage [16], and antioxidant activities [17]. Despite these studies, no report has been made to date on *C. intermedia* to serve as a possible source of depigmentation compounds to alleviate the excessive production and or formation of melanin [18].

Thus, the aim of this study was to perform an investigation on a crude ethanolic extract *of C. intermedia* plant material as well as its column chromatographic fractions for possible anti-tyrosinase activity in an *in vitro* system. The various bioactive fractions against tyrosinase (VII-IX) were also structurally investigated for the presence of phenolic compounds. The work was further extended to investigate the correlation between *in vitro* antioxidant capacities of the bioactive fractions VII-IX with the skin depigmentation activity demonstrated.

## 2. Experimental Methods

### 2.1. Chemicals and Reagents

The chemicals L-ascorbic acid, 2,2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH), 2,2-azino-di-3-ethylbenzthiazoline sulphonate (ABTS), (+)-catechin hydrate, 7,12-dimethylbenz[α] anthracene (DMBA), fluorescein sodium salt, gallic acid, quercetin dihydrate, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), 2,4,6-tri[2-pyridyl]-s-triazine (TPTZ), kojic acid, L-tyrosine, tyrosinase enzyme from mushroom, ethylenediaminetetraacetic acid (EDTA), trichloroacetic acid (TCA), butylated hydroxytoluene (BHT), thiobarbituric acid (TBA) were purchased from Sigma-Aldrich (Steinheim, Germany), while 4-dimethylaminocinnamaldehyde (DMACA) and Folin Ciocalteu's phenol reagent were purchased from Merck (Hohenbrunn, Germany).

### 2.2. Plant Material and Extract Preparation

Commercially available "green" (unfermented) *Cyclopia intermedia* plant material was a gift from Rooibos Limited (Clanwilliam, South Africa). The dried plant material (200 g) was first extracted at room temperature (25°C) with chloroform (10% m/v) for 24 hours (repeated twice) in an Erlenmeyer flask, under rotation and filtered through Whatman No. 4 filter paper. The residual plant material was further extracted with 100% ethanol as described for chloroform, filtered, and evaporated to dryness at 40°C and 150 rpm under reduced pressure in a rotary evaporator, resulting in a crude ethanolic extract coded GHE.

### 2.3. Thin Layer Chromatographic (TLC)-Guided Column Fractionation

5.0 g of GHE was applied on a silica column (24 cm × 16 cm), fractionated into nineteen fractions (200 mL each) by column chromatography (CC) using a gradient of ethyl acetate-hexane system (0%, 10%, 30%, 50%, 70%, 100%) respectively. The fractions were developed on TLC (silica gel/Hexane: ethyl acetate 7:3). After removal of the mobile phase, the plate was sprayed with vanillin sulphuric acid for the detection of polyphenolic compounds. Fractions with similar profiles under ultra violet (UV: 254; 366 nm) and when sprayed with vanillin sulphuric acid, were combined resulting in nine (I-IX) combined fractions.

### 2.4. High Performance Liquid Chromatography (HPLC) Determination of Individual Phenolic Compounds

An Agilent 1200 HPLC system consisting of a quaternary pump, autosampler, online degasser, column oven and diode-array detector with Chemstation 3D LC software was used for HPLC-DAD analysis. Peak identity was determined by means of retention time, UV spectra characteristic at 280 nm and comparison to that of authentic polyphenol standards previously isolated from honeybush [13]. The Synergi Max-RP (C12-bonded silica, 4 μM, 80Å) column was used with separation carried out at 30°C with a flow rate of 0.8 mL/min using gradient of 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B). The gradient started at 100% solvent A for 1 minute and changed to 28% B over 22 minutes in a linear way. It then went to 40% of solvent B over 50 seconds and a wash step of 1.5 minutes at 100% of solvent B, followed by re-equilibration to initial conditions for 4 minutes. The injection volumes for both samples (VII-IX) and the standards were 20 μL.

### 2.5. LC-MS Analysis for Further Confirmation of Peak Identity of GHE and Fractions

The identity of peaks as observed in the HPLC chromatogram was further confirmed by comparing their mass spectra with that of pure standard compounds (purchased from Sigma-Aldrich, South Africa). A Waters Synapt G2 quadrupole time-of-flight mass spectrometer used for the LC-MS analysis, fitted with a Waters Ultra pressure liquid chromatograph and photo diode array detector was used for the analyses. Separation was achieved using a Waters HSS T3, 2.1 × 150 mm column with 1.7 μm particles with same mobile phase as in HPLC. The flow rate was 0.3 mL/min with injection volume 1 μL. The instrument was operated with an electrospray ionization probe in the negative mode with sodium formate used for calibration and leucine encephalin infused in the background as lock mass for accurate mass determinations.

### 2.6. Colorimetric Tyrosinase Inhibition Assay

A previous method described was adopted with slight modification [19]. All samples (GHE, I-IX) were dissolved in dimethyl sulphoxide (DMSO) to a stock solution of 1 mg/mL

(w/v). Further dilutions were done with 50 mM sodium phosphate buffer (*p*H 6.5) for all working solutions to the concentrations of 400; 200; 100; 50 & 25 µg/mL. In the wells of a 96-well plate, 70 µL of each sample working solution was combined with 30 µL of tyrosinase (from mushroom, 250 Units/mL in sodium phosphate buffer) in triplicate. After incubation at room temperature for 5 minutes, 110 µL of substrate (2 mM L-tyrosine) was added to each well and the reacting mixture was then incubated for 30 minutes at room temperature. The enzyme activity was determined by measuring the absorbance at 490 nm using plate reader. The percentage of tyrosinase inhibition was calculated as follows:

$$[(A - B) - (C - D)] / (A - B) \times 100 \quad (1)$$

## 2.7. Total Polyphenol and Flavonoid Content of GHE and Bioactive Column Fractions

The total polyphenol content of the ethanol extract and its column bioactive fractions against tyrosinase (VII-IX) was determined using the established Folin Ciocalteu method [20]. A volume of 25 ml of the extract GHE and fractions VII-IX was incubated in a clear 96-well flat bottom plate for 5 min with 125 ml freshly prepared 0.2 N. Folin Ciocalteu's phenol reagent was thereafter 100 ml 7.5% sodium carbonate was added and incubated for 2 hours. The absorbance at 765 nm was measured on a plate spectrometer (Multiskan, Thermo Electron Corporation) and total polyphenols calculated using the standard gallic acid in 10% ethanol. Results were expressed as mg gallic acid equivalents per gram extract.

The flavanol content of GHE, VII, VIII and IX was determined colourimetrically at 640 nm and spectrophotometrically at 360 nm, respectively in accordance to the previous methods [21, 22]. A freshly prepared 0.05% DMACA solution was made by dissolving DMACA in 8% HCl prepared in methanol. The samples (50 mL) were incubated in a clear 96-well flat bottom plate with 250 mL DMACA solution for 30 min.

For the flavonol assay, a solution of 0.1% HCl prepared in 95% ethanol was made. The samples (12.5 ml) were incubated in a clear 96-well flat bottom plate with 12.5 mL 0.1% HCl-ethanol solution and 225 mL 2% HCl for 30 min. The absorbance was measured on a plate spectrometer (Multiskan, Thermo Electron Corporation) and flavanols and flavonols calculated using the standards catechin in methanol and quercetin in 95% ethanol, respectively. Results were expressed as mg standard equivalents per gram extract.

## 2.8. Total Antioxidant Capacity of GHE and Bioactive Fractions VII-IX

The oxygen radical absorbance capacity (ORAC) was determined according to the fluorometric method described [23]. A 36 ng/mL fluorescein and a 2.5% AAPH radical solution were freshly prepared in 75 mM phosphate buffer (pH 7.4). The reaction was initiated by incubating 12 mL of the samples in a black 96-well flat bottom plate with 138 ml fluorescein solution and 50 mL AAPH solution. The decrease in fluorescence was then measured every 5 minutes for 2 hours on a plate fluorometer (Fluoroskan, Thermo Electron Corporation). The ORAC values were determined by comparing the sample curve to the standard curve obtained for trolox. Results were expressed as mmol trolox equivalents per gram extract.

The ferric reducing antioxidant power (FRAP) and the trolox equivalent antioxidant capacity (TEAC) was determined according to the spectrophotometric methods described [24, 25]. For the FRAP assay, the samples (10 mL) were incubated in a clear 96-well flat bottom plate in 300 mL FRAP solution (250 Mm sodium acetate in acetic acid, pH 3.6; 0.83 mM TPTZ in 40 mM hydrochloric acid; 1.67 mM Iron (III) chloride hexahydrate) at 37 °C for 30 minutes. The reaction was then measured at 593 nm on a plate spectrometer (Multiskan, Thermo Electron Corporation) and the FRAP value determined by comparing to the standard ascorbic acid. Results were expressed as mmol ascorbic acid equivalents per gram extract. For the TEAC assay, an ABTS radical solution was prepared by incubating 7 mM ABTS with 2.42 mM potassium peroxodisulfate in the dark overnight. The solution was diluted with ethanol to an absorbance of 2. The samples (25 mL) were incubated in a clear 96-well flat bottom plate in 300 mL ABTS radical solution for 30 min. The TEAC value was then determined by measuring the reaction at 734 nm on a plate spectrometer (Multiskan, Thermo Electron Corporation) and compared to the standard trolox in ethanol. Results were expressed as mmol trolox equivalents per gram extract.

## 2.9. Inhibition of Fe (II)-Induced Microsomal Lipid Peroxidation Assay

A method described [17] with a few modifications was adopted. The reaction mixture contained microsomes (1 mg of protein/mL in 0.01M potassium phosphate buffer; pH 7.4, supplemented with 1.15% KCl). The sample stock solutions (GHE & VII-IX) were prepared in methanol (1 mg/mL, w/v), while the working sample solutions were prepared in 0.01M potassium phosphate buffer; PH 7.4, supplemented with 1.15% KCl diluted to 400; 200; 100; 50 and 25 µg/mL concentrations. 100 µL of each sample (working solutions) were dissolved in potassium phosphate buffer and pre-incubated with 500 µL microsomes at 37°C for 30 minutes in a shaking water bath. 200 µL of KCl-buffer were added to the mixture, followed by 200 µL of a 2.5 mM ferrous sulphate solution and incubated at 37°C for 1 hour in a shaking water bath. The reaction was terminated with 10% trichloroacetic acid (TCA) solution (1 mL) containing 125 µL butylated hydroxytoluene (BHT, 0.01%) and 1mM ethylenediaminetetraacetic acid (EDTA). Samples were centrifuged at 2000 rpm for 15 minutes, 1 mL of supernatant was mixed with 1 mL of 0.67% thiobarbituric acid (TBA) solution. The reaction mixture was then incubated in a water bath at 90°C for 20 minutes and the absorbance were measured at 532 nm using plate reader. The percentage inhibition of TBARS formation relative to the positive control was calculated by:

$$[(A_{control} - A_{sample}) / A_{control}] \times 100 \quad (2)$$

### 2.10. Statistical Analyses

The green ethanolic extract of honeybush (GHE), and column fractions I-IX data was first analysed by linear regression analysis using Graphpad prism 5.00 software Inc. Fe (II)-induced microsomal lipid peroxidation data was downsized to only the bioactive fractions of significant tyrosinase inhibition < 100.00 µg/mL with such data analysed by Graphpad prism 5.00. All other data (antioxidant capacities, total polyphenol and flavonoid content) were expressed as equivalent of each standard. A *p* value of 0.05 was considered statistically significant.

## 3. Results and Discussion

### 3.1. Chromatographic and Mass Spectrometry Consideration

Gradient of silica column fractionation carried out on the crude ethanolic extract of *C. intermedia* (GHE) after the removal of lipophilic contents by chloroform, yielded nine (9) combined fractions (I-IX). The combination of certain fractions was guided by their individual characteristics on TLC as well as their% inhibition of tyrosinase at the threshold concentration 100.00 µg/mL.

Peaks identification of the active fractions VII-IX and GHE by HPLC resulted in the identification of notable polyphenols: mangferin, hesperidin, eriodyctol, naringenin & kaempferol previously reported to be present in honeybush [17] in addition to a considerable number of unidentified peaks. The mass spectrum obtained further suggested the presence of these polyphenols which are in agreement with previous data on the existence of these polyphenols in C. *intermedia* [17]. Meanwhile, unidentified peak at a retention time of 9.71 min ([M-H] 153.02) is tentatively assumed to be protocatechuic acid with fragment ions ([M-H] 152; 122), but such product ions could not be identified on the basis of LC-ESI-MS characteristics. Other peaks with MS characteristics assumed to be phenolic acids ([M-H] 177.02 & 187.10), and other unidentified polyphenols ([M-H] 269.05, 285.04, 285.21 & 301.07) needed further chromatographic isolation and purification, followed by spectroscopic analysis for their complete structural elucidation.

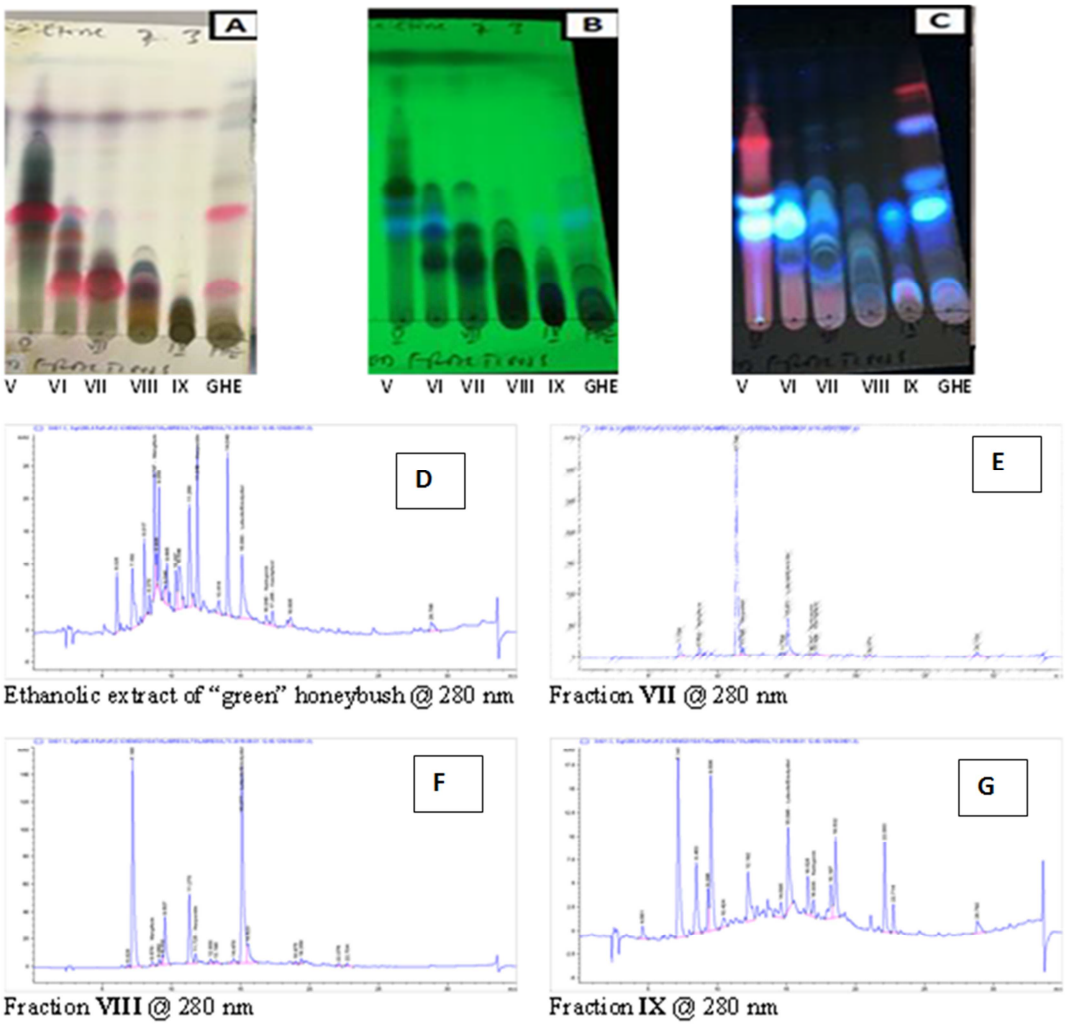


***Figure 1.*** *Chromatographic profiles of "green" honeybush ethanolic extract (GHE). Notations: A = TLC chromatogram when sprayed with vanillin sulphuric acid; B = TLC chromatogram when viewed under UV lamp 254 nm; C = TLC chromatogram when viewed under UV lamp 366 nm; D-G =HPLC chromatograms of GHE, VII, VIII & IX respectively. Abbreviations: GHE = ethanolic extract of "green" C. intermedia; V-IX = column fractions.*

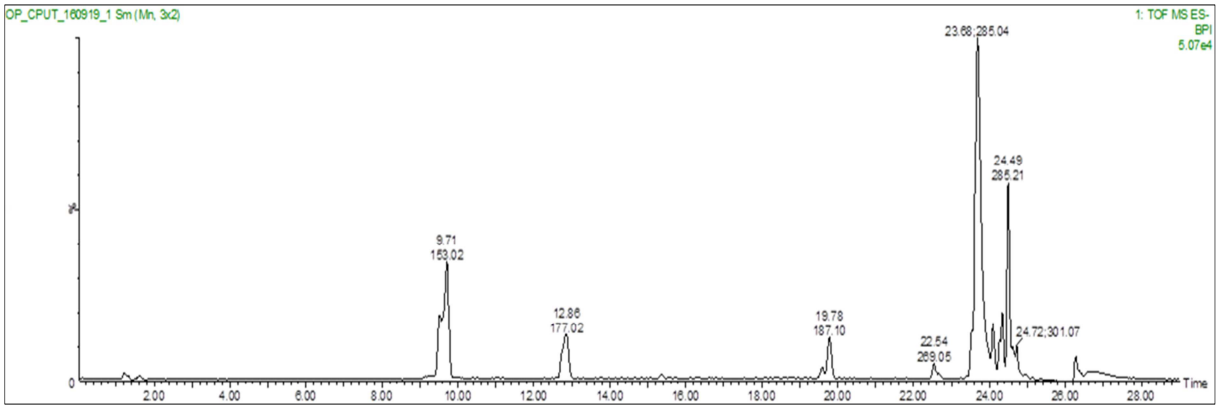


***Figure 2.*** *Mass chromatogram of column fraction VIII of "green" ethanolic extract of C. intermedia (GHE).*

## 3.2. Biological Activities Consideration

In South Africa, honeybush is traditionally useful in the treatment of skin related diseases including rashes [16]. In this study, we evaluated the tyrosinase inhibitory and antioxidant capacity properties of the column fractions obtained as well as the crude ethanolic extract of "green" *C. intermedia* plant material. The inhibition of these fractions on tyrosinase activity using L-tyrosine as substrate is presented as $IC_{50}$ in Table 2. The results showed that fraction VIII exerted a considerable level of *in vitro* mushroom tyrosinase inhibition ($IC_{50}$ 28.125 µg/mL), which is just 5-fold higher than that of kojic acid, a well-known tyrosinase inhibitor used for reference, while other column fractions (VII & IX) and the crude extract (GHE) are 15-fold higher than the control drug.

The total polyphenol content (Table 1) of GHE extract (134.30 mg GAE/g) was significantly ($p < 0.05$) higher than individual column fractions VII-IX (38.99; 131.49 & 57.36 mg GAE/g) respectively. Similar trend was observed for flavanol and flavonol content of GHE higher than its corresponding column fractions. Our notable observation was the highest flavonol and total polyphenolic contents of VIII (131.49 mgGAE/g & 314.35 mgQE/g) respectively than other column (VII & IX), which may likely be responsible for the anti-tyrosinase activity demonstrated by VIII [6, 18].

Three different complementary assays were used to determine the antioxidant capacity of the bioactive samples against tyrosinase at the cut-off concentration 100.00 µg/mL. The most active fraction (VIII) against tyrosinase ($IC_{50}$ 28.125 µg/mL) was found to contain high TEAC (9903.21 µmol TE/g), FRAP (6146.38 µmol AAE/g), ORAC (3218.72 µmol TE/g), while ethanolic extract (GHE) with anti-tyrosinase ($IC_{50}$ 84.062 µg/mL) showed TEAC (2078.80 µmol AAE/g), FRAP (6144.50 µmol TE/g), & ORAC (3948.11 µmol TE/g). Results from the FRAP (r = 0.974) and TEAC (r = 0.970) assays correlated with each other while neither of the FRAP nor TEAC correlated with ORAC (r = 0.927). This may suggest that the mechanism of antioxidant activity demonstrated by the tested samples proceed via electron transfer in both neutral (TEAC) and acidic (FRAP) media. The correlation between total antioxidant capacities and the contents of total polyphenol and flavonoid components of *C. intermedia* were also evaluated. The results revealed that there was not a linear correlation between antioxidant properties and total polyphenol or flavonoids contents of the examined extracts. The lack of a linear correlation between these assays might result from existence of other class of natural compounds in *C. intermedia* ethanolic extract synergistically responsible for the antioxidant properties demonstrated herein.

The structural arrangement of flavonols and possibly other phenolic content present in VIII may be responsible for diverse biological activities, as previously described in the literature to include its ability to chelate with trace metals [12] and exhibit a mild anti-tyrosinase inhibition [6]. The contribution of other unknown prominent peaks (Figures 1 & 2) to this activity demonstrated by VIII may not to be ruled out, nevertheless, we suggest that highest flavonol and polyphenolic contents recorded in column fraction VIII may be chiefly responsible for the anti-tyrosinase activity demonstrated.

***Table 1.*** *Total polyphenol, flavonoid content and Total Antioxidant capacity of green honeybush and its column chromatographic fractions.*

| | Total polyphenol content and flavonoid | | | Total antioxidant capacity | | |
|---|---|---|---|---|---|---|
| **Extract/fraction** | **Total polyphenol content (mg GAE/g)** | **Flavanol (mg CE/g)** | **Flavonol (mg QE/g)** | **FRAP (µmol AAE/g)** | **TEAC (µmol TE/g)** | **ORAC (µmol TE/g)** |
| GHE | 134.44 ± 90.01 | 49.05 ± 31.91 | 346.72 ± 171.01 | 6144.50 ± 39.06 | 2078.80 ± 130.01 | 3948.11 ± 211.16 |
| VII | 38.99 ± 18.8 | 47.16 ± 125.17 | 37.16 ± 36.61 | 1052.55 ± 19.99 | 399.50 ± 24.80 | 987.46 ± 09.11 |
| VIII | 131.49 ± 89.11 | 41.20 ± 18.32 | 314.35 ± 153.11 | 6146.38 ± 5.72 | 9903.21 ± 161.73 | 3218.72 ± 90.14 |
| IX | 57.36 ± 134.01 | 40.93 ± 53.03 | 16.50 ± 18.83 | 5621.36 ± 33.88 | 1196.10 ± 77.43 | 603.72 ± 53.18 |

Values in columns are means ± SD of three determinations (n = 3). Abbreviations: GAE = gallic acid equivalents; CE = catechin equivalents; QE = quercetin equivalents; FRAP, ferric reducing antioxidant power; TEAC, trolox equivalent antioxidant capacity; ORAC, oxygen radical absorbance capacity; AAE = ascorbic acid equivalents; TE = trolox equivalents; GHE = "green" honeybush; VII-IX = column fractions.

***Table 2.*** *Inhibitory activities of the "green" honeybush ethanolic extract and active fractions.*

| Fraction | Inhibition | |
|---|---|---|
| | Tyrosinase ($IC_{50}$ µg/mL) | LPO ($IC_{50}$ µg/mL) |
| GHE | 84.062 | 138.590 |
| VII | 77.500 | 326.011 |
| VIII | 28.125 | 260.173 |
| IX | 84.062 | 262.887 |
| Kojic acid | 5.125 | NA |

$IC_{50}$ values were calculated using linear regression of GraphPad Prism 5.00 software ($p \leq 0.5$). Abbreviations: GHE = ethanolic extract of "green" *C. intermedia*; VII-IX = column fractions; LPO: Fe (II)-induced microsome lipid peroxidation.

## 4. Conclusion

Occurrence of peak at retention time 9.71 tentatively correlate to the MS profile of protocatechuic acid ([M-H] 153.02) which has not been reported previously from *C. intermedia*. The current data from this study suggest *C. intermedia* to have potential uses in both the cosmeceutical and food industries. Future investigations is proposed to include large scale extraction as to isolate some of the unknown/non-detectable peaks on HPLC, and to further explore them for possible use in skin depigmentation and other skin pigment-related ailments/diseases. In this regards, a pilot study from our research group showed fraction VIII to also have anti-melanoma activity in B16 cells, but these results will be further explored (unpublished data).

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## Conflict of Interests

The author declare no conflict of interest.

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